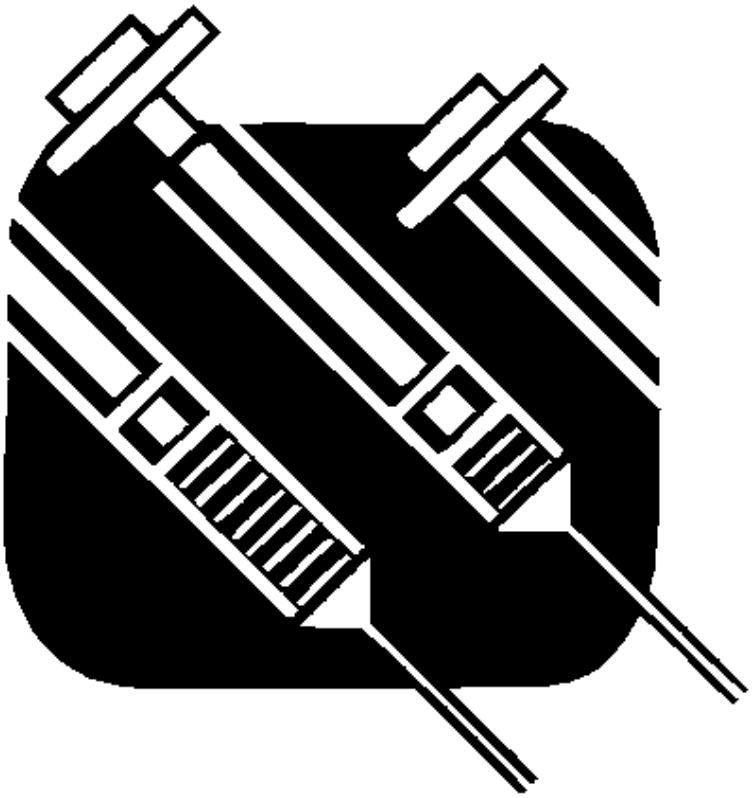
Search for correlates of protection



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Abstract

Nowadays a wide variety of vaccines exist to prevent diseases. However, the correlates of protection in immunisation are an unresolved issue. This hinders the development of new vaccines and improvement of current vaccines. To measure the efficacy of newly developed vaccines, several methods can be used, such as determining the antibody titer, measure the CTL activity and determine the cytokine production. However, all of these methods have their limitations. The antibody titer does not correlate to the efficacy of the vaccine. Moreover, titers can be measured in various ways, making them incomparable. Also, only the humoral response is measured. This makes it limited to asses the complete protection. To indicating the strength of the cellular response, CTL activity can be determined. This is however, an inconvenient method to use and contradicting results were found in human studies when correlating it to the efficacy of vaccines. The role of cytokines during an immune response is still not clear and therefore would not be a good parameter to determine the efficacy. Another aspect with regard to immunisation, are the influence of factors such as age, route of delivery and lifestyle. There exact influence, however, needs to be determined yet. Therefore, more research is necessary to determine the specificity of parameters and the affecting factors. Identifying the optimal correlates of protection per vaccine is recommended. This would be easier if the different methods were optimised and standardised. Using several methods will extract the mechanism behind immunisation, making it easier to develop new vaccines.

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1. Introducing vaccines

1.1 General mechanism

Nowadays a wide variety of vaccines exists to prevent outbreaks of diseases. Although many experiments are conducted to develop good vaccines, the mechanism behind immune priming is still largely unknown. This makes it difficult to assess efficacy of newly developed vaccines and compare them with already existing vaccines. Fast and efficient development of vaccines is important, because immunisations against several infectious diseases such as Human Immunodeficiency Virus (HIV), Herpes simplex virus and malaria are still lacking. Other vaccines need improvement, such as the influenza vaccine, which provides only weak protection in elderly. Some vaccines are deficient in safety demands and have many adverse effects. Therefore, it is important that a representative method is designed to allow assessment of efficacy of vaccines, so that vaccines can be improved and new vaccines can be tested.

In this review, I will compare the different correlates of protection of vaccines. First, the general mechanism of vaccines will be discussed and what types are being developed. Then I will mention what types of parameters are used in animal models and in human trials. I will also focus on a few factors that play a pivotal role in inducing immunisation. Finally, I will compare the different findings between animal and human research and discuss which parameter should be used when determining the efficacy of a vaccine.

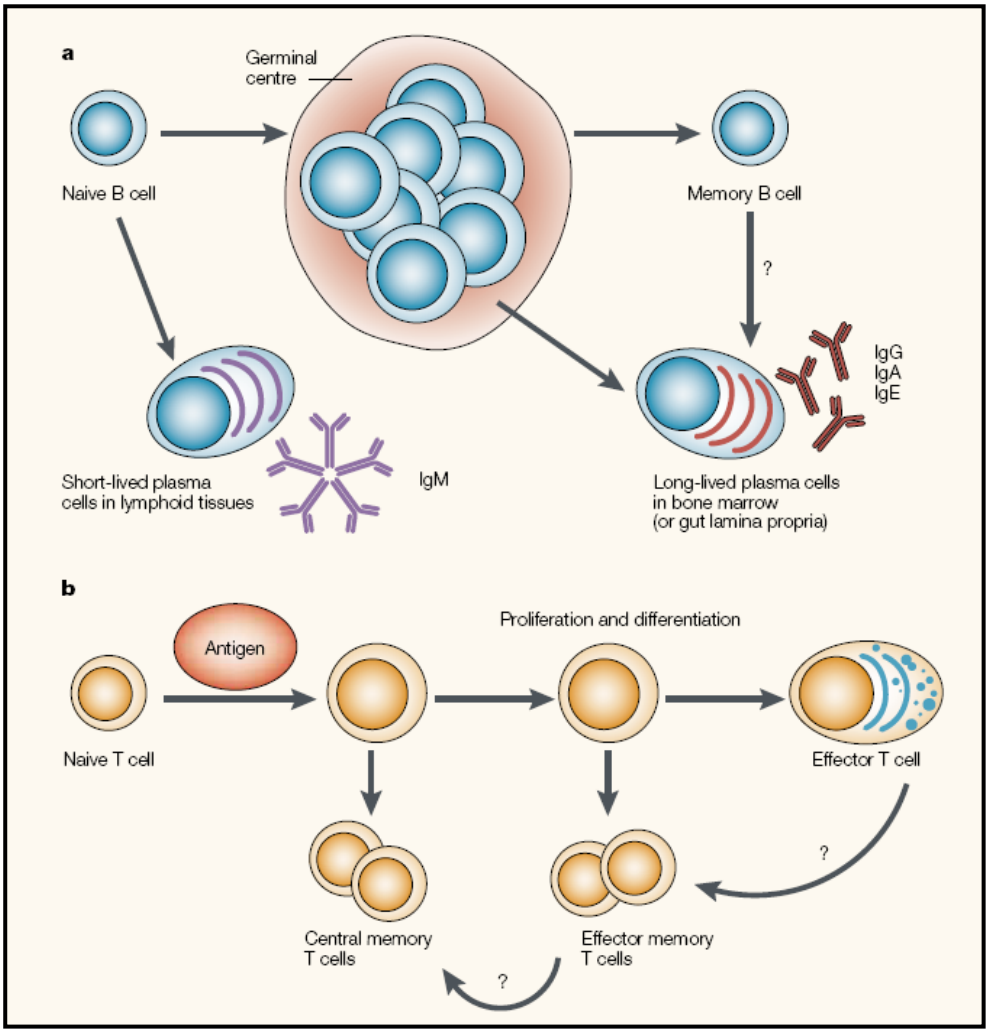

Figure 1. The Development of memory B and T cells. a] Production of plasma cells and memory B cells in the germinal centre. b] Production of effector T cells and memory T cells. Adapted from Gray 2002.

When immunisation was performed for the first time in the 18th

century, the mechanism behind it was unknown. Since then, many experiments have been conducted to unravel this phenomenon and several kinds of vaccines have been produced. Generating protection against a pathogen is difficult because the immune system is complex and many factors can influence the immune response. Protection can be offered by a humoral or cellular response, driven by the cytokines secreted from T-helper (T_h, $CD4^+$) cells. These cells activate naive B-cells and T-cells, which transform into effector cells or memory cells, as shown in figure 1.

For a protective and specific immune response to occur, the pathogen needs to be presented to effector cells. This task is being performed by antigen presenting cells (APC), and especially dendritic cells (DC) are specialized in doing so. DCs are present in high numbers in the derma and gut where pathogens can enter the host. They reside there in an immature status and after encountering an antigen, they migrate to lymph tissue. During this transition, DCs change their morphology from antigen take-up to antigen presenting cells. In the lymph tissue, they activate T-cells and B-cells. DCs are therefore thought to be key players in the immune system. They are activated by the presence of antigens and co-stimulatory signals coming from other cells such as innate immune cells and $CD4^+$ cells. Follicular DCs and T_h cells attract B-cells into the germinal centers (GC) of lymph nodes, in which they differentiate into specific plasma cells or memory cells (Plotkin, Orenstein, Vaccines). Antibodies are important in the clearance of a pathogen, however, memory is needed to sustain the protection and therefore it is necessary to elicit a T-cell response. The transition of B-cells to effector cells or memory cells can only occur when T_h-cells are activated and enter lymph nodes. T_h-cells increase the affinity and specificity of antibodies from B-cells, thereby improving the clearance of a pathogen. Therefore, activation of T_h-cells is necessary to elicit memory B cells and specific antibodies (Plotkin, Orenstein, Vaccines).

Next to B-cells, T_h-cells can also activate cytotoxic T-cells ($CD8^+$, CTLs). When a $CD8^+$ cell is activated, it can differentiate into an effector cell or a memory cell. Memory T cells can survive lifelong without antigen exposure. When the pathogen re-enters, the memory cells start to proliferate and differentiate to elicit a fast and strong immune response. T_h1 cells secrete IFN-γ and TNF-α and activate CTLs and macrophages, whereas T_h2 cells secrete IL-4, IL-5 and IL-13 directed against extracellular pathogens. Both pathways can stimulate B-cells for more antibody production, but only follicular T_h-cells can stimulate GC B-cells to increase affinity and induce memory cells (Plotkin, Orenstein, Vaccines). To successfully clear a pathogen, the right amount of each arm of immune response should be elicited. Proteins from pathogens can skew the immune response via the receptors on innate immune cells, such as pattern recognition receptors (PRR) on neutrophils. These receptors recognize repetitive patterns of eukaryotic cells and thereby become activated and secrete cytokines which can skew the T_h response to a T_h1 or T_h2 side. To balance this process, T regulatory cells (Tregs) are activated. It is thought that Tregs are formed when the cells are presented with an antigen but do not receive signal 2, therefore are not activated to differentiate into an effector cell. Tregs are thought to down regulate the immune response in order to prevent massive reaction to harmless antigens. However, more research is necessary to completely understand the differentiation and mechanism behind Tregs.

In this review, several studies on the efficacy of vaccines were analysed. These studies were conducted on either animal or human models. Table 1 represents a small selection of different studies on immunisation in animal models. This shows the diversity of assessing efficacy of vaccines among the studies. The results of the animal studies will be discussed in chapter 2. In chapter 4, the studies on human models will be discussed. In chapter 5, they will be compared to each other.

Tabel 1. Overview of several studies which measure efficacy of vaccines in animal models.

Reference	Animal model	Measure efficacy		Vaccine type
		Quantitative	Qualitative	
Koff, Johnson et al. 2006	Indian rhesus macaques	Viral load in sera		Life attenuated SIV virus
Dittmer, Brooks et al. 1998	Mice (B10.A x A/Wy)	Viral load in plasma	Survival and splenomegalic	Live attenuated Friend virus
Puri, Weyand et al. 2000	Mice (CD-1, female)	Titer antibody (IgG) in sera	Presence of microspheres in derma	Muramyl dipeptide loaded ovalbumin microspheres
Skene, Doidge et al. 2008	Mice, female	Colony forming assays, titer antibody in sera		Purified antigens of *h. pylori* bacteria
Obeid, Stanley et al. 1996	Mice (CBA/J)	Titer antibody (IgG 1, 2ab, 3) in sera, affinity antibody	Survival	Measles virus, TTB chimeric peptide
Mills, Ryan et al. 1998	Mice (C57BL/6)	Colony forming assay, cytokine assay (IFN-y, IL-5), Titer antibody (IgG 1,2) in sera and lungs	Prevalence disease in children	Acellular and whole cell *Bordetella pertussis* bacteria
Elamanchili, Lutsiak et al. 2007	Mice (BalB/C, male)	Stimulator capacity of DCs, cytokine assay (IL-1 to IL-13, GM-CSF, IFN-y, TNF-a) clonal expansion of T-cells	Morphology DC's (MHC II, CD86, CD40) and T-cells (CD44, CD62L, CD69)	BLP25
Barry and Johnston 1997	Mice	Titer antibody, Viral load		Cytomegalovirus promoter with α-1 antitrypsin (DNA vaccine)
Spellberg, Ibrahim et al. 2008	Mice (BalB/C, female)	Titer antibody (IgG 1, 2) in sera	Survival	Purified proteins of *saccharomyces cerevisiae* (vaccine), infected with *Candida albicans* (yeast)
Schnurr, Chen et al. 2005	Human DCs, $CD8^+$ and $CD4^+$ cell lines	INF-y production of cells		NY-ESO-1 protein purified from *E. coli* bacteria
Zuckermann, Garcia et al. 2007	Swine (White line York x Landrace, female)	Viral load in lungs, tonsils and sera, titer antibody, IFN-y and IL-10 production,	Clinical progress	Modified live PRRS (porcine reproductive and respiratory syndrome) virus and killed virus
Gramzinski, Millan et al. 1998	Aotus l. lemurinus monkey	Titer antibody (IgG) in sera		Hepatitis B surface antigen DNA vaccine with *E. coli* promoter
McCluskie, Brazolot Millan et al. 1999	Mice (BalB/C, female), Rhesus monkey	Titer Antibody (IgG 1, 2) in plasma (monkeys), in sera (mice)	Lysis activity of CTL	*Hepatitis B* surface antigen DNA vaccine with *E. coli promoter*
Heinemann, Dillon et al. 2004	Mice (BalB/C, female)	Titer Viral infected cells	Body weight of mice, pathology of lungs	Vaccinia virus VVE2 β-gal loaded DC's
Todryk, Kelly et al. 1998	Mice (SJL)	Lymphocyte and T- cell proliferation, titer	Antigen presentation of DCs.	Polypeptide fragment 3 from *Streptococcal SA I/II*

		antibody (IgG 1, 2) in sera		
Williamson, Eley et al. 2000	Mice (Porton outbred, female)	Titer antibody ((IgG 1,2,3) in sera, spleen and BAL, Titer viral load in spleen, liver and lungs	Survival	Fraction 1 and V antigens of *Yersinia Pestis* bacteria and killed whole cell formulation
Ulmer, Deck et al. 1994	Mice (BalB/C, male and female), Rhesus monkeys (male and female) and African green monkeys (male)	Titer antibodies in sera,	Survival, Cr release of CTLs	HA and NP (V1JHA and V1JNP) genes from *Influenza* in DNA plasmid

1.2 Developed vaccines so far

Since the knowledge on immunisation has improved, new vaccines have been developed to prime the immune system for pathogens. Immune priming is now expanding to other areas of medicine in ways that the immune system can also be primed against tumours and auto-immune diseases. The focus in this review however, will be on vaccines targeted against pathogens, to limit the complexity.

The ideal vaccine would be efficient, easy to administer and profitable to produce. However, most vaccines do not comply with these criteria. Because the mechanisms behind immunisation are not completely known, it is difficult to determine the role of each cell type in the immune response. Consequently, assessing the correlates of protection is also difficult. Preferably, a vaccine should activate $CD4^+$ and $CD8^+$ cells, thereby elicit a strong immune response and induce high antibody titers and high lysis activity of CTLs. This should lead to a decline in prevalence of a disease after immunisation, reflecting the protection of a vaccine. Whether the protection and the immunogenic parameters are truly representatives of each other must be demonstrated. Therefore, it is difficult to asses the correlates of protection for vaccines.

leeftijd	vaccinatie 1	vaccinatie 2
0 maanden (geboorte)	HepB0*	
2 maanden	DKTP-Hib1†-(HepB‡)	Pneu1§
3 maanden	DKTP-Hib2-(HepB)	Pneu2
4 maanden	DKTP-Hib3-(HepB)	Pneu3
11 maanden	DKTP-Hib4-(HepB)	Pneu4
14 maanden	MenC‖	BMR1¶
4 jaar	DKTP5	
9 jaar	DTP6	BMR2

*Vaccinatie tegen hepatitis B: alleen voor zuigelingen van HBsAg-positieve moeders.
†Acellulair combinatievaccin tegen difterie, kinkoest, tetanus, poliomyelitis en *Haemophilus influenzae* type B. Tot 2005 bestond de kinkhoestcomponent daarin uit hele bacteriecellen.
‡Hepatitis B-vaccin wordt toegediend aan kinderen met een ouder uit een land waar hepatitis B midden- of hoogendemisch vóórkomt en aan kinderen van HBsAg-positieve moeders.
§Vaccinatie tegen *Streptococcus pneumoniae* serotype 4, 6B, 9V, 14, 18C, 19F en 23F voor kinderen geboren vanaf 1 april 2006.
‖Vaccinatie tegen meningokokken type C.
¶Vaccinatie tegen bof, mazelen, rodehond.

Figure 2. Vaccination Scheme maintained in the Netherlands. Adapted from van der Maas, David et al. 2007.

Currently, the most efficient technique to prime the immune system is by using live attenuated strains of a pathogen (Dittmer, Brooks et al. 1998; Koff, Johnson et al. 2006). They induce strong immune responses presumably via PRRs which identify pathogen associated proteins (Plotkin, Orenstein, Vaccines). This activates the immune system and thereby induces a strong response resulting in memory cell formation. PRR activation is also used in non-live vaccines; however, due to the lack of replication of non-live vaccines, they induce a more limited response. Therefore, it is important to use adjuvants to stimulate the reaction

and to induce memory build-up. These two forms of vaccines have been used for decades, but the mechanisms behind it are still not completely unravelled. Nonetheless, they are used worldwide in infants, children and adults.

In the Netherlands, a vaccination scheme is maintained to prevent outbreak of epidemic diseases, as shown in figure 2. All infants repeatedly receive a DKTP-Hib, BMR, *pneumococcal* and *meningococcal* type C vaccine. The first immunisation is given at the age of 2 months and the last is given at 9 year of age[1]. The DKTP-Hib vaccine consists of killed virus of *poliomyelitis* and toxoids of *B. pertussis*, *C. diphtheriae*, *C. tetani* and *H. influenzae* type B. The BMR vaccine consists of live attenuated virus of *Myxovirus parotiditis, pseudomyxo* virus and *rubella* virus. These vaccines are nationally provided by the government since 1957 and have not been modified since then[2]. Although new techniques are being explored, they have not been able to exceed their successors in efficacy or safety.

Although these vaccines seem to work, other vaccines fail to induce protection. One problem which needs to be tackled is the lack of T cell responses after immunisation. Some vaccines are efficient in eliciting high antibody titers, but protection remains low, presumably due to lack of cellular memory. A new technique that is being developed is genetic immunisation e.g. DNA vaccination. This technique is invented to resolve the absence of T cell response of some vaccines. The theoretical approach of DNA vaccination is that the DNA encodes a protein of a pathogen. This is injected into the host, which transfers the DNA into its cells. There the DNA is transcribed and translated, and presented via MHC I receptors. This will be recognized by T cells and an immune response is induced. Because DNA on itself is not pathogenic, the DNA is delivered in a bacterial vector with repeated motifs to stimulate the innate immune system. Additional promoters can be inserted into the vector to increase DNA transcription (Shedlock and Weiner 2000). This is one new immunisation technique that is developed to tackle pathogens which do not induce a sufficient T cell response to elicit memory. Several advantages this vaccine has is that it is more temperature stable and can be produced at relative low costs, making this vaccine better available for third world countries (Shedlock and Weiner 2000). Drawbacks associated with this vaccine, which need to be investigated further before introducing it on a wide scale, are the induction of anti-DNA autoimmune responses and tumorgenesis.

Several other techniques exist to prime the immune system, such as epitope enhancement. By increasing the affinity for MHC molecules, APC cells will present more antigens. Improving affinity for MHC can also increase the activation of CTLs by T_h cells. This can skew the immune response towards a T_h1 response, via CD40L interaction and altered cytokines secreted by T helper cells (Berzofsky, Ahlers et al. 2001).

More ways to induce immunisation is by optimising intracellular processing of epitopes in DCs. One article reveals techniques to increase the presentation of antigens and thereby induce more memory when the immune response is increased. This can be done via increasing the affinity of peptides for TAP transporters and MHC I molecules. This will lead to faster and more presentation of epitopes which will

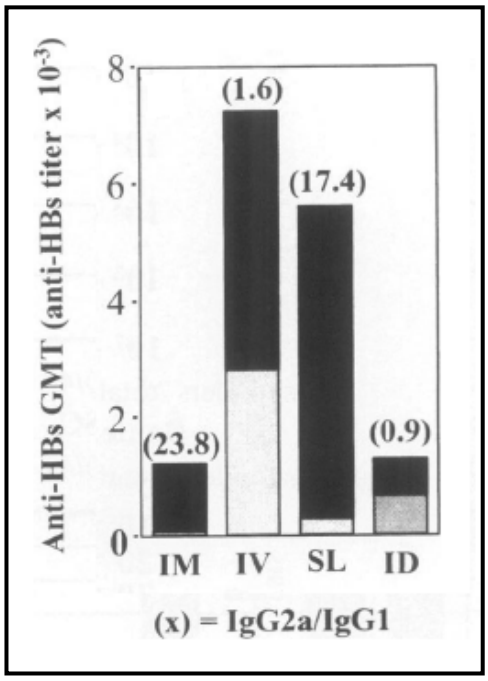

Figure 3. IgG titers of immunised mice after different immunisation methods. Mice were immunised against Hepatitis B. Intramuscular and sublingual injections of antigen show a more T_h1 response. Adapted from McCluskie, Brazolot Millan et al. 1999.

induce a higher response (Del Giudice 2003).

Although these techniques sound very promising, it is difficult to assess their efficacy and compare them to other vaccines. Because the exact mechanism behind immunisation is still elusive, it is difficult to find an efficient parameter to measure the efficacy and memory build-up of vaccines. Therefore, I will review the most common methods that have been used so far and discus problems which have to be tackled to find immune parameters that correspond with protection.

2. Immune correlates of protection in animal studies

2.1 Antibody titer

The most common way to determine the efficacy of a vaccine is measuring the amount of specific antibodies produced upon vaccination. It reveals the activity of the B-cells and can be correlated to the clearance of certain bacterial pathogens (Mills, Ryan et al. 1998). However, it fails to correlate to the protection induced by vaccines against other pathogens. Therefore, the specificity of this parameter needs to be assessed.

Box: Comparing the differences in antibody measurements among studies.

Comparing the antibody titers of different studies is difficult because all measurements are done in different manners. Some are done by ELISA measurements of serum on 96 wells plate coated with antigen (Puri, Weyand et al. 2000; Spellberg, Ibrahim et al. 2008), which can also be subdivided in peptide specific or total antigen antibodies (Obeid, Stanley et al. 1996). Next to this, the analysis of the data can differ, as the titer can be determined by taking the mean absorbance value in the linear dilution range, or by taking the last serum dilution that is more than 2 standard deviation above the mean for negative control samples (Puri, Weyand et al. 2000; Spellberg, Ibrahim et al. 2008). Furthermore, there is a difference between avidity and affinity of antibodies, and not al studies make a distinction between those two. This is important in determining the specificity of an immune response. The time between measuring antibodies and immunisation also differs between the studies, ranging from 30 days (Barry and Johnston 1997) to 8 weeks (McCluskie, Brazolot Millan et al. 1999). Also, antibodies can be measured in samples taken from tail vein puncture or by retro orbital puncture (McCluskie, Brazolot Millan et al. 1999). The latter will contain venous blood and also tissue fluid, which is different in composition than samples taken from the tail vein (Van Herck, Baumans et al. 2001). The pCO2 and Na^+ are higher in orbital puncture, and pH and K^+ are lower. The anesthesia necessary to perform such sampling can also influence the blood. Next to these differences, also different genotypes of mice were used. These differences can be reflected in the results. Therefore, it is impossible to specify the correlate of protection of antibody titer.

By measuring antibodies, the type of immune response can be determined, as certain factors influence the production of antibodies. For instance, the cytokines which B-cells receive determine what type of antibodies they produce. The isotype of antibodies are markers for a T_h1 or a T_h2 response (Deenick, Hasbold et al. 2005). For example, TGF-β induces the production of IgG2b and IgG3, and IL-4 induces the production of IgG1 and IgE, which are all markers for a T_h2 response (Plotkin, Orenstein, Vaccines). INF-γ induces the production of IgG2a, which is a marker for a T_h1 response. However, cytokines do not only affect antibody production, but also

influence the survival and differentiation of other immune cells. These cells can skew the immune response to a T_h1 or a T_h2 response and thereby indirectly affect B-cells and the antibody production (Deenick, Hasbold et al. 2005).

Not only can the nature of the antigen affect the immune response, but also other factors influence the antibody production. One such factor is the delivery mode. The different ways to administer the antigen can skew the immune response. This was found when mice were immunised against Hepatitis B (HBV). An intradermal (ID) injection skewed the response towards T_h2 while a more T_h1 response was found with intramuscular (IM) and sublingual (SL) injections, as shown in figure 3 above (McCluskie, Brazolot Millan et al. 1999). More factors have shown to influence the T_h1/T_h2 balance, such as the type and dose of antigen, number and spacing of immunisations, type of host and adjuvant (McCluskie, Brazolot Millan et al. 1999). Several of these factors will be discussed later.

The amount of specific antibodies elicited after immunisation has been positively related to survival of mice when challenged with the yeast *Candida albicans* (Spellberg, Ibrahim et al. 2008). However, it was found that younger mice of 4 to 6 weeks of age produce higher titers of antibodies than older mice of 8 to 10 weeks (Barry and Johnston 1997). This implies that age is related to the reactivity of immunisation. More interestingly, the amount of antibodies elicited after immunisation also differed among the genotypes of mice (Barry and Johnston 1997). This shows that multiple factors can influence the production of antibodies.

Comparing the results of these studies is difficult due to the fact that different methods or different analyses are used, as is explained more elaborately in the textbox. All these differences can be reflected in the results, making it impossible to compare them. This makes it difficult to asses the correlation of this parameter to the humoral response upon immunisation. Therefore, methods should be more aligned, so that this parameter can be more specified.

2.2 CTL activity and cytokine production

Other ways to measure the efficacy of vaccines is by measuring the activity of effector cells. A CTL response is important when immunising against intracellular pathogens, therefore it is useful to measure CTL activity as a parameter to determine the efficacy of such a vaccine. One major shortcoming of newly developed vaccines is that they sometimes fail to elicit a cellular response, which is also necessary to induce proper memory. Therefore, it is important to measure this response, even though it is less convenient to measure compared to the antibody titer. CTL activity is measured commonly by isolation of CTLs from the spleen and measuring the activity via Cr release from target cells using ELISA. The amount of activated CTLs correlates with the strength of the T_h1 response upon immunisation.

Because CTL lysis activity is an important arm of protection to fight off intracellular pathogens, it is essential to develop it early in life. It can already be induced by immunisation in mice which are just 2 weeks old (Siegrist, Saddallah et al. 1998). Also very low amounts of antigen, from 1 μg, can be enough to induce CTL lysis in mice and provide protection after challenge with a pathogen (Ulmer, Deck et al. 1994) To elicit a strong CTL response, T_h cells are necessary, or at least the CTL binding proteins of those cells (Shirai, Pendleton et al. 1994).

The amount of CTLs elicited differs depending on the nature of the vaccine. A study was conducted on mice which were immunising against Malaria with three different antigens: Ty virus-like particles (VLPs) and whole modified vaccinia virus Ankara (MVA), both expressing an epitope from *P. berghei,* and a DNA antigen. The CTL responses were measured after immunisation (Gilbert, Schneider et al. 2002). The DNA antigen induced the least amount of CTLs, the MVA and VLPs induced more CTLs. However, using the antigens

heterologously to prima and boost the immune response increased the amount of CTLs even more. Also higher CTL lysis activity was found when boosters and primers were used heterologously (Degano, Schneider et al. 1999). However, increasing the variety to more than two does not have a more beneficial effect. These results were not expected, as it is generally known that the immune response is stronger when the antigen is encountered for the second time. Therefore, it is odd that a stronger immune response was induced when two different antigens were used instead of one antigen. This shows that immune responses are complex and more factors must be involved.

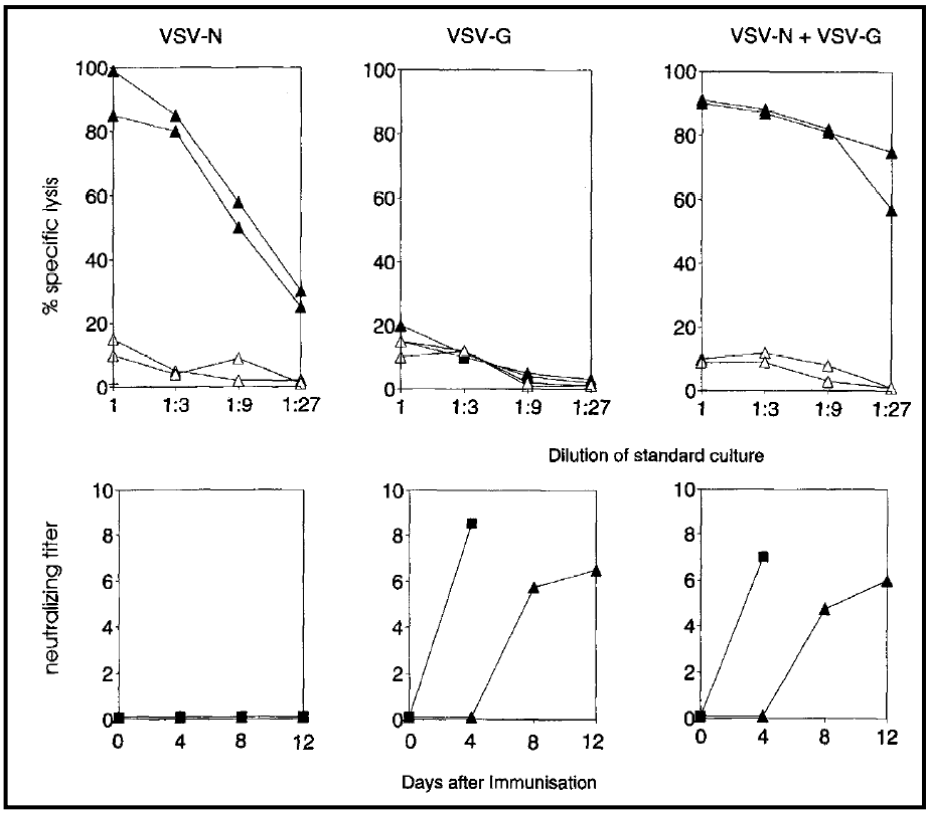

Figure 4. CTL activity and antibody titer of mice immunised with vesicular stomatitis virus (VSV-N), glycol-protein of VSV (VSV-G) or both. Upper panels show lysis activity of VSV-N (close triangles) or mocked (open triangles) transfected cells. Lower panels show IgM (square) or IgG (triangles) antibody titer. In the VSV-N model, CTL activity is present, but no antibodies are formed. In the VSV-G model, antibodies are formed, but no CTL activity is present. Using both models, CTL activity and antibodies are present. Modified from Bachmann, Hengartner et al. 1994.

Another odd observation was made when two different vaccines were used to immunise mice against vesicular stomatitis virus. The vaccines either consisted of a glycoprotein (VSV-G) or a nuclear protein (VSV-N) of the virus. As shown in figure 4, it is possible to induce a cellular response without inducing antibody production and vica versa, depending on the type of antigen used to immunise (Bachmann, Hengartner et al. 1994). This shows that antigens from one pathogen can induce either a humoral or a cellular response. Therefore, determining more than one immune parameter in one experiment can give more insight in the effects of an immunisation.

To gain more knowledge about the immune response, the cytokine secretion from $CD4^+$ cells can be measured. This might clear up the discrepancy found between the elicited antibodies and CTLs, as T_h1 and T_h2 cytokines secreted from DCs and $CD4^+$ cells determine which pathway will be activated. Therefore, measuring the environmental factors of immune cells may reveal the whole picture of the response. However, this is difficult as the production of cytokines can be influenced by several factors.

One of those factors is the type of antigen used to immunise. When comparing a plasmid vector antigen and a HBV surface antigen, the former induced more INF-γ and IL-12 (T_h1), whilst more IL-4 and IL-5 (T_h2) was produced when the latter was used (Xiao-wen, Shu-han et al. 2005; Elamanchili, Lutsiak et al. 2007). Similar results were found when pigs were immunised with a modified live vaccine or a killed virus vaccine. The latter induced

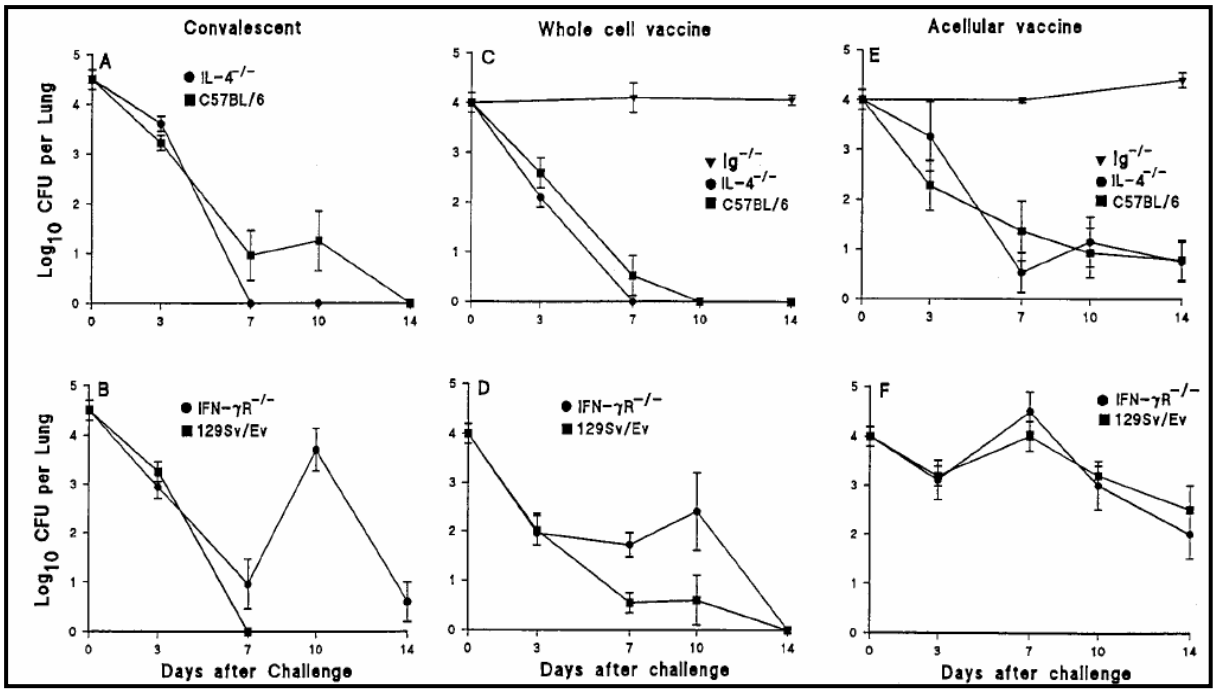

Figure 5. Clearance of *B. Pertussis* after immunisation of wildtype (C57BL/6 and 129Sv/Ev), Ig, IFN and IL-4 KO mice. Convalescent indicates clearance of pathogen without immunisation. Immunised IFN$^{-/-}$ and IL-4$^{-/-}$ mice still clear the pathogen after challenge, Ig$^{-/-}$ mice do not. Adapted from Mills, Ryan et al. 1998.

higher spontaneous production of IL-10 by peripheral blood mononuclear cells (PBMC). Also, the spontaneous release of IFN-γ was increased (Zuckermann, Garcia et al. 2007). The differences in produced cytokines can bias the immune response towards one T_h pathway, which influences the protectiveness of the host.

To investigate the exact role of cytokines in protection upon immunisation, cytokine production was measured in different knock-out mouse models. Immunising IFN-γ deficient mice against *Candida albicans* and then challenging the mice reveals that without IFN-γ, mice are not able to defend themselves against yeast infections (Spellberg, Ibrahim et al. 2008). However, IFN-γ$^{-/-}$ mice are able to combat a bacterial infection, although not in the same amount as the wild type mice (Mills, Ryan et al. 1998). IL-4$^{-/-}$ mice are less sensitive for infection compared to IFN-γ$^{-/-}$ mice, the survival after a yeast infection is similar to that of wild type mice, the clearance of bacteria was even better than the wild type mice, as shown in figure 5 (Mills, Ryan et al. 1998; Spellberg, Ibrahim et al. 2008). Next to this, IFN-γ$^{-/-}$ mice kept producing IL-5 after respiratory challenge with *B. pertussis*, even though IL-4$^{-/-}$ and wild type mice only produced T_h1 cytokines (Mills, Ryan et al. 1998). This may mean that it is easier to induce a T_h2 response and that the natural state of the immune response is skew to that pathway.

However, this does not seem to be the case when comparing the isotype switching in B-cells, which is regulated by cytokines. It was found that IL-4 and IFN-γ can inhibit LPS induced switching to IgG3 (Deenick, Hasbold et al. 2005). Putting cytokines together revealed a hierarchy of isotype switching, where IFN-γ is the most dominant versus IL-4 and TGF-β, and IL-4 is dominant over TGF-β (Deenick, Hasbold et al. 2005). This would imply that T_h1 cytokines are more dominant over T_h2 cytokines and can prevent the switching to T_h2 antibodies. Although this was only seen in the isotype switching of B-cells, it does introduce contradicting result when compared to survival as mentioned above.

In conclusion, the results discussed show that the role of cytokines in the immune response is not clear yet, which makes them an unreliable parameter when determining the

efficacy of vaccines. This becomes even more obvious when considering the fact that cytokines do not necessarily have a one to one relation with the T_h1/T_h2 responses, as CTL activity can be induced in the presence of T_h2 cytokines (Siegrist, Saddallah et al. 1998).

Taken together, CTL activity can be a good parameter to measure the cellular response to certain pathogens. Moreover, the results from CTL assays from the different studies are easier to compare than antibody titers due to the similarity among the methods and techniques, which makes the parameter reliable. However, it is too limited to determine the efficacy of vaccines, because protection can also be offered via the humeral response.

2.3 Alternative correlates

Other ways to determine the efficacy is by measuring the actual clearance of the pathogen. The survival or welfare of animals after infection can also be measured. This will show directly the actual protection of immunisation. However, it will reveal little about the mechanisms behind the protection.

Clearance can be measured by taking samples from lung or stomach and determine the amount of colony forming units (CFU) that form when the samples are spotted on plates (Mills, Ryan et al. 1998; Skene, Doidge et al. 2008). One study immunised mice against *H. pylori* and measured the CFU in the stomach upon infection (Skene, Doidge et al. 2008). Comparison of intragastrically immunisation via a tube or subcutaneous (SC) injection revealed no difference in CFU between the two methods. However, the amount of IgG did differ between the two methods, the latter induced higher IgG titers. This shows that discrepancies can occur between the actual protection and the immune response behind this.

Another downside of measuring clearance of a pathogen is the biased results which can be obtained when measuring the clearance locally. This became apparent when the viral load in sera, lungs and tonsils was compared after challenging immunised pigs with porcine reproductive and respiratory syndrome virus (PRRSV). The viral load was undetectable in lungs and sera, but was still present in the tonsils, even after immunisation, albeit at a lower level than the unvaccinated control (Zuckermann, Garcia et al. 2007). This implies that the virus was cleared systemically and from the site of infection, but apparently the virus was still present in some tissues of the body. This could lead to biased conclusions, as a limited picture is obtained from the results. Therefore, it is important to choose corresponding sites to measuring clearance of pathogens.

Another parameter which can be looked at is the survival of animals after challenging them with pathogens (Ulmer, Deck et al. 1994; Williamson, Eley et al. 2000; Spellberg, Ibrahim et al. 2008). Furthermore, factors such as weight loss indicating illness of the animals after challenge can be documented. This is an approach which will reveal the protection of immunisation, although it reveals little about the mechanism behind it. Also, it cannot be used in human studies.

The alternative methods presented above can be used as indicators, but they have their limitations. Since little information is gathered about the mechanisms behind the immunisation, it might lead to false conclusions. Therefore, it is advisable to use more correlates to determine the efficacy.

3. Factors influencing immune response

3.1 Dose of vaccine

When examining the parameters, it became apparent that several factors influence immunisation, thereby affecting the efficacy of vaccines. An attempt was made to distil

general mechanisms behind these factors, in order to understand their effects on immunisation. Therefore, some important factors will be discussed in the following chapter.

One influencing factor is the dose of antigen that is used. It is known that a low dose of antigen elicits a T_h1 response (Plotkin, Orenstein, Vaccines). However, to optimise protection it is convenient to induce both arms of immunisation. It depends on the pathogen what type of response is more important, but to optimise the humoral response, T_h-cells are necessary to increase affinity of the antibodies and induce memory. Therefore, it is important to understand what type of immune response is induced at different doses. This was investigated by a research group which immunised mice against West Nile virus and determined the titer of different IgG isotypes (Lieberman, Clements et al. 2007). The IgG2a/IgG1 ratio increased as the dose of antigen increased. This shift of antibody type illustrates a switch of the immune response from a T_h2 to a more T_h1 response, as shown in figure 6. This reveals that the dose of antigen has a significant effect on the type of immune response.

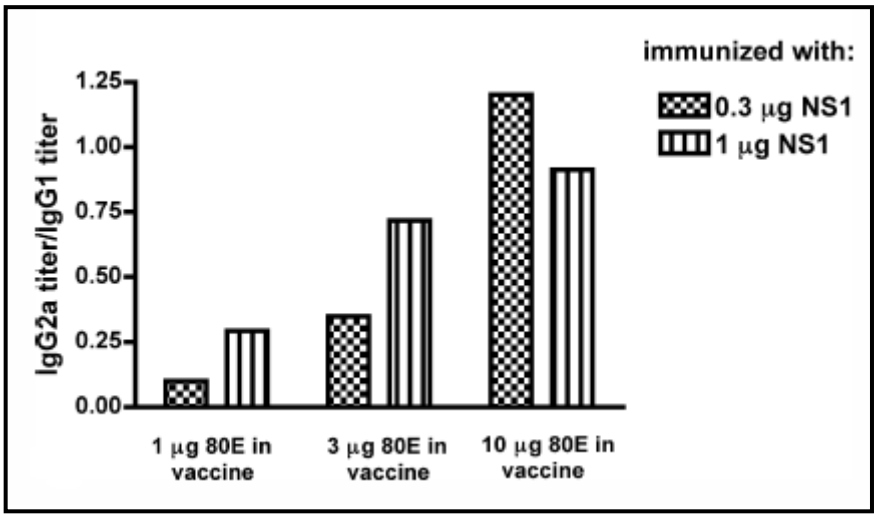

Figure 6. IgG2a/IgG1 ratio after immunising mice with different amount of antigen. The ratio increases concomitant with the increased dose of vaccine. This reveals a shift from a T_h2 to a more T_h1 response. Adapted from Lieberman, Clements et al. 2007.

Moreover, the spacing between the doses can also influence the immune response. It is generally assumed that short spacing, 1 to 2 weeks, yields high antibody magnitude and persistence, but longer spacing, 1 to 2 months, yields live long protection. It is thought that this is due to high affinity follicular B-cells that may give rise to memory B-cells, which are not formed when the antigen is presented for a short time. To sustain live long protection however, boosting is probably necessary. This does not seem to apply for live attenuated vaccine, which can induce antibody responses that last for decades in the absence of antigen exposure (Plotkin, Orenstein, Vaccines).

More research is done on limiting the amount of doses needed and it seems that new vaccines with higher antigen concentrations need fewer doses to elicit more antibodies with the right properties (Williamson, Eley et al. 2000). This shows that the nature of the vaccine is correlated with the doses needed to induce protection. Moreover, using a good adjuvant can increase the immunisation against pathogens, also resulting in fewer doses.

3.2 Adjuvants

The use of adjuvants is very important to elicit a strong response against antigens. It is one of the main determinants for the initial inflammatory reaction (Plotkin, Orenstein, Vaccines). Although Alum is the only adjuvant allowed in human practice, much research is focussed on finding other adjuvants. The main reason for this is to find adjuvants that can interfere with the immune system in such a way that a highly specific and strong protection is elicited. Adjuvants can have different targets and functions in immunisation. They can influence the

delivery of the antigen, induce co-stimulatory or induce T_h1 or T_h2 immune responses (O'Hagan, MacKichan et al. 2001).

The different effects of adjuvants became apparent when different adjuvants were tested on mice immunised against measles (Obeid, Stanley et al. 1996). A synthetic peptide of measles virus was used as antigen and combined with different adjuvants. The antibody titers were measured and compared to the survival of the animals after challenge. The results were highly variable. The immunisation with IL-2 did not induce any protection or correlating antibodies after challenge, even though IL-2 plays a prominent role in the cellular immune response. Alum induced the highest survival rate after challenge, although it did not yielded the highest amount of antibodies. Freunds' incomplete adjuvant (FIA) did induce the highest amount of antibodies, but not the highest survival. This reveals that the amount of antibodies is not necessarily correlated to protection. However, it does reveal that adjuvants have a large effect on the outcome of immunisation and that this effect differs per adjuvant.

To increase the immune protection, it is also possible to use multiple adjuvants. This attempt was made in a study on mice which were immunised against Malaria. However, the titer of antibodies or CTL activity did not increase when two or three adjuvants were used, compared to using only one adjuvant, as shown in figure 7 (Hui and Hashimoto 2008). This shows that the increased immune response induced by adjuvants can not be added up.

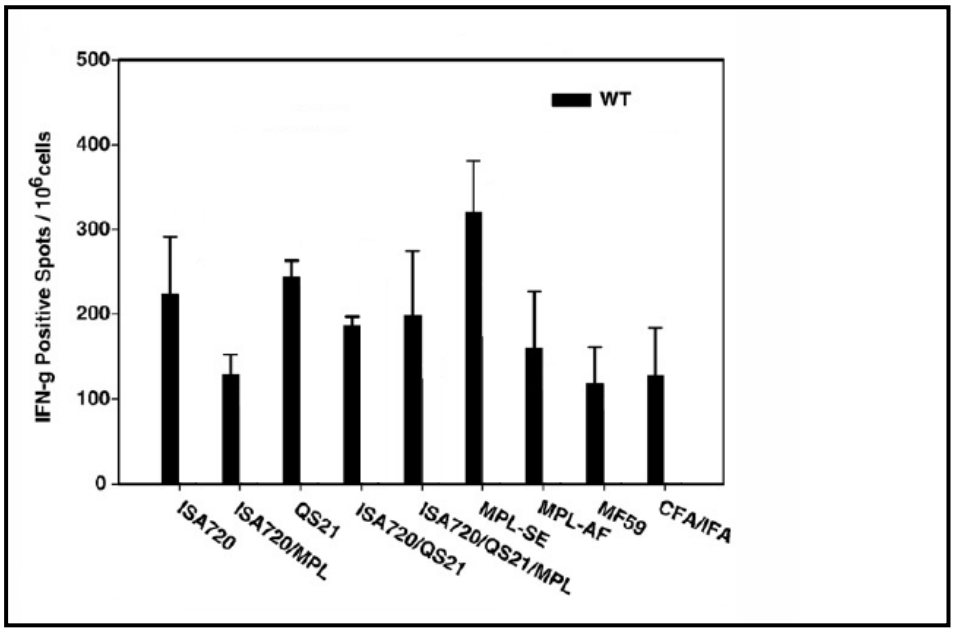

Figure 7. Elispot of INF-γ production of splenocytes of mice challenged with antigen after several immunisations. Using multiple adjuvants did not increase the CTL activity compared to using one adjuvant. Modified from Hui and Hashimoto 2008.

On the other hand, they did found that the formulation of the vaccine and adjuvant affects the immune response. Immunising with an emulsion of adjuvant and antigen yielded the highest amount of antibodies, compared to aqueous and oil formulations (Hui and Hashimoto 2008). It was thought that this is due to efficient internalizing and presenting of the antigen from the emulsion by APCs. This shows that the mechanism behind immunisation with adjuvants may differ among the adjuvants.

Next to increasing the immune response, adjuvants can also skew the immune system. Several adjuvants are known to skew to a T_h1 response, such as CpG DNA, Mycobacterium vaccae and Quil A saponin (O'Hagan, MacKichan et al. 2001; Dredge, Marriott et al. 2002). By activating APCs in such a manner that they secrete T_h1 cytokines skews the immune response. T-cells can also be influenced directly via signalling by CD40L, which also induces a T_h1 response and therefore could be used as an adjuvant in viral vaccines (Tripp, Jones et al. 2000). However, it should be noted that the mechanism of these adjuvants is not completely understood and can cause some serious side effects. For example, it has been shown that CpG

can induce autoimmunity (O'Hagan, MacKichan et al. 2001). Therefore, more research needs to be done before it can be used in human population studies.

3.3 Route of delivery

Next to the type of antigen, the route of delivery can also influence the efficacy of vaccines. There are many different ways to deliver a vaccine and some of them have been investigated thoroughly (Gramzinski, Millan et al. 1998; Todryk, Kelly et al. 1998; McCluskie, Brazolot Millan et al. 1999; Puri, Weyand et al. 2000). Although most vaccines are delivered traditionally by IM or SC injection, other ways have some advantages. Mucosal administration is easier and delivers the antigen on the site where most pathogens initially establish infection (O'Hagan, MacKichan et al. 2001). However, uptake of antigen can be hampered by the mechanical and chemical barriers of the host, which are already broken when antigen is administered via injection. This can influence the efficacy of vaccines.
The importance of this became apparent when different routes for immunisation were used to immunise mice against HBV. Different non-injecting routes such as intranasal inhalation (INH), intrarectal and ocular anointment, and oral feeding were used and different injecting routes such as intraperitoneal (IP), ID, intravenous (IV) and IM injection. All the non-injecting routes did not elicit any antibodies and CTL activity, except for INH which did showed weak CTL activity (McCluskie, Brazolot Millan et al. 1999). Immunisation against *H. pylori* via the nasal route and oral route revealed that the former route induced more clearance of pathogens (Skene, Doidge et al. 2008). This could be due to the fact that more APCs are reached in the airways, as this is a common entry for pathogens. Other injecting routes can induce higher protection, as more APCs are reached to present the antigen.

Activating APCs in the skin such as Langerhans cells, which are better antigen presenters than peritoneal macrophages, can induce higher protection. Comparing IP injection with SC injection revealed that more antibodies with higher affinity are produced in the latter. Also, survival after challenge was higher with SC delivery, as shown in figure 8 (Obeid, Stanley et al. 1996). DCs are abundantly present in the skin, where most infections start, consequently injecting ID would activate the most DCs. ID injection induced a higher amount of antibodies compared to SC injection. Next

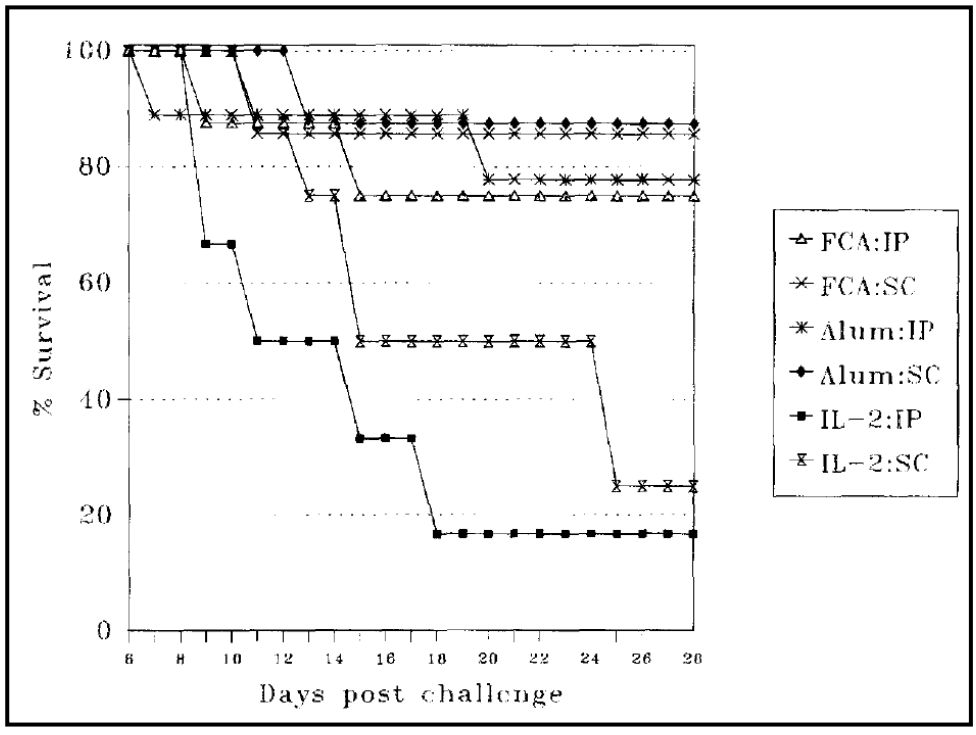

Figure 8. Survival of mice immunised via SC or IP injection and challenged with measles virus. SC injection of the vaccine resulted in higher survival. Adapted from Obeid, Stanley et al. 1996.

to this, more antigen was translocated to the lymph nodes (Puri, Weyand et al. 2000). Also, a lower dose of antigen can be used when injecting ID to elicit the same response compared to SC injection.

Delivery of antigen on micro scale also influences the immune response. When antigen was delivered by linking it to immune complexes, antigen presentation could be blocked by protease inhibitors. This was not the case for delivering antigen with an adjuvant (Schnurr, Chen et al. 2005). Therefore, it seems that cross-presentation may be altered when the antigen is delivered by immune complexes compared to adjuvants. This reveals that the antigen is processed on different manners, which can affect the presentation skills of DCs, thereby changing the immune response.

Next to the DC activity, CTL activity is also modified with different mode of delivery. It was found that two doses of gene gun (GG) administration resulted in weaker CTL activity than one dose of IM or ID injection (McCluskie, Brazolot Millan et al. 1999). On the other hand, another study found that IV immunisation against VSV yielded excellent CTL responses, but IP and SC immunisation did not (Bachmann, Hengartner et al. 1994). Moreover, it was found that only ID injection offered complete protection after challenging with malaria (Gilbert, Schneider et al. 2002). These contradicting results make it difficult to evaluate the impact of the different delivery modes of vaccines. It could be possible that the different delivery routes target different cells; therefore some routes will work better than other routes, depending on the natural infection route of the pathogen. However, for most vaccines the specific mechanism behind the protection is not known, therefore, it is not possible to determine the most effective route.

To increase the complexity even more, the injection equipment can also influence the efficacy of vaccines. Using a “needle free Biojector” induced higher humoral responses than a needle or syringe, probably due to more widespread of the antigen (Gramzinski, Millan et al. 1998). This was applicable for both IM and ID administering, although the ID route overall induced more antibodies than IM delivery. This shows that next to the site of administering, the type of equipment may also reach different cells, thereby influencing the immune response.

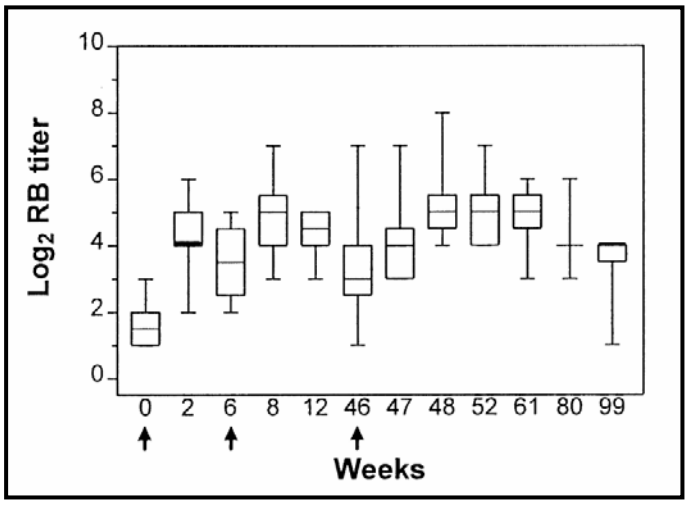

Figure 9. Opsonophagocytic activity of PMNs taken from 10 adults after 3 times of immunisation. Arrows indicate time of immunisation with meningococcal vaccine. After a third boost the opsonopgagocytic activity increases again. Adapted from Naess, Aarvak et al. 1999.

Therefore, it is important to realise that route of delivery can influence the efficacy of vaccines and should be taken into consideration when producing and applying vaccines. Apparently, when different cells are reached by the antigen, different responses are elicited. Therefore, the route of delivery should be considered for every vaccine, as the most effective immune response differs per antigen. However, it is also important to keep in mind that easier administering can increase the scale and compliance of patients, thereby saving more people.

4. Efficacy of vaccines in human population studies

4.1 Correlates of protection in human studies

Now that the different correlates of protection were studied on animals, it is interesting to determine which of these are used in human studies. Although a large scale of methods is used to measure efficacy of vaccine in animal models, there is not much variety in the methods of testing vaccines in human populations. Next to this, a wide range of vaccines are studied in animals, but the regime of vaccines used in humans remains constant. It is difficult to create new vaccines without side effects and with low cost-effectiveness. Moreover, the safety demands for testing the candidate vaccines are high, which hampers the implementation of vaccines to humans. Next to this, there are limited parameters that can be measured in human populations, making it difficult to determine the efficacy. Due to practical and economical limitations, the antibody titer is often the only correlate of protection which is measured. On the contrary, it is possible to do long follow up studies, which provide some information about the duration of antibody titers. However, the mechanisms behind it stay elusive.

As measuring the antibody titer has been a frequently used method to determine the efficacy of immunisation, some encouraging observations have been made. For instance, using a good adjuvant can decrease the amount of antigen needed by threefold (Hehme, Engelmann et al. 2004). It was also found that half of the amount of antigen that is advised by authority is still enough to elicit the criteria amount of antibodies to yield protection in students younger than 20 years (Baldy, de Lima et al. 2004). This also applies for the number of doses used to immunise. No difference in titer of antibodies was found when infants of 2 months were immunised twice or trice for pneumococcal infection (Goldblatt, Southern et al. 2006). The titer of HBV antibodies of children under the age of 5 years was also not influenced by the number of doses or injection site of immunisation (Whittle, Jaffar et al. 2002). This can be very important for administration of vaccines in case of pandemics.

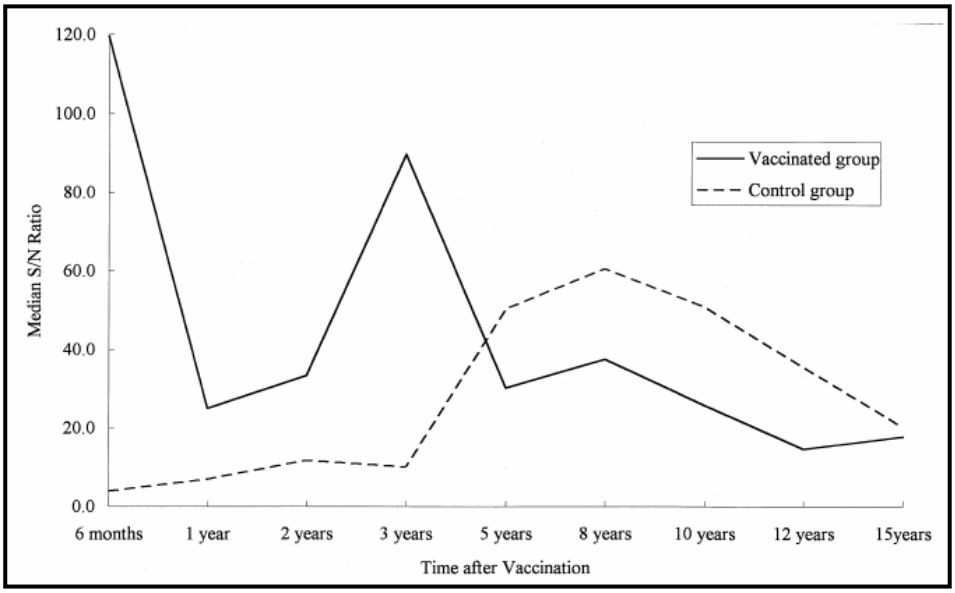

Figure 10. Amount of Hib specific antibodies after 15 year of follow up of Chinese infants, starting at 6 months of age. After 5 years the amount of antibodies of the vaccinated group declined to the same level as for the control group, even though the protection was maintained. Adapted from Liao, Li et al. 1999.

However, it became apparent that no differences were found when infants were immunised, but differences did come up when adults were immunised. When an extra booster was administered against Hib several months after the last immunisation, the amount of antibodies significantly increased. Also opsonophagocytic activities increased, as is

shown in figure 9 above (Naess, Aarvak et al. 1999). This shows that altered doses do not necessarily influence the amount of antibody or protection in infants, but it can have an influence in adults. Somehow aging seems to make a change in the mechanism behind immunisation, as will be discussed later.

One factor adding to the complexity of immunisation is the lifetime of the antibody producing cells. The exact lifetime of antibody generating plasma cells is still under debate, ranging from several days to several months (Radbruch, Muehlinghaus et al. 2006). However, when the antibodies of 8 child diseases in adults with a mean age 37 years were measured, the titer changed only slightly in 15 years of follow up (Amanna, Carlson et al. 2007). This shows that the production of antibodies remains constant for many years, indicating that plasma cells have to persist at the same level also. However, this does not necessarily have to correlate, as protection can remain even though the antibody titers have waned. This was seen in a 15 year follow up study of Chinese infants of 3 to 36 months (Liao, Li et al. 1999). Although the efficacy of the vaccine was about 89% after 15 years (efficacy means that the chance for contracting HBV was 89% smaller after immunisation), the mean antibody titer declined to the same level as the control after 5 years, as shown in figure 10 above.

One possible explanation for this could be the avidity of antibodies. A study on toddlers of 2 to 3 years of age revealed that 40 weeks after immunisation against meningococcal B, the avidity of antibodies had increased, while the amount of antibodies had declined (Vermont, Van Dijken et al. 2002). This indicates that protection can be offered by small amounts of antibodies which have high avidity. It has been found for pneumococcal immunisation that higher avidity of antibodies offered more protection in adults of 18 to 43 years (Usinger and Lucas 1999). However, the exact mechanism behind the protection offered via the humoral response needs to be determined.

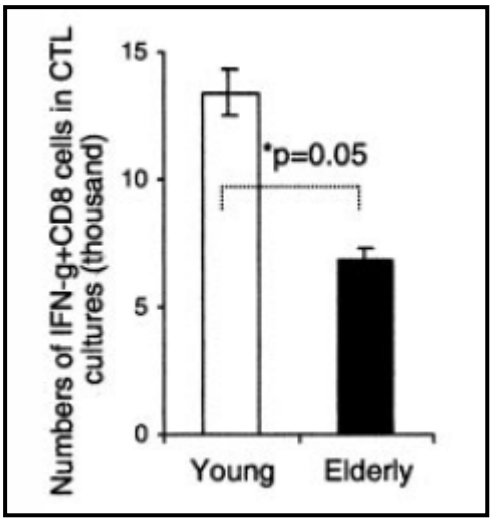

Figure 11. Numbers of influenza activated CTLs in young people and elderly. Elderly show diminished CTL activation compared to young people. Adapted from Deng, Jing et al. 2004.

Another interesting fact found during studies on infants, was the long lasting protection after immunisation. Infants can build up persistent memory for at least 15 years, as was seen in the 15 year follow up study (Liao, Li et al. 1999). Another study found that immunisation against HBV yielded protective antibody titers lasting for 10 years, in both infants and adults (Zanetti, Mariano et al. 2005). Also cellular protection can remain, as the protection of a BCG vaccine was determined to be 55% after 60 years (Aronson, Santosham et al. 2004). Subjects in this study were vaccinated at a mean age of 8 years, showing that immunisation of children can last almost throughout a life time.

Although a high variety in methods determining the humoral response was found in animal studies, not many differences were found in human trials. Therefore, it does not affect the results as much. In animal models, various methods are used to obtain blood samples, such as retro orbital puncture, tail vein puncture or cardiac puncture (Todryk, Kelly et al. 1998; McCluskie, Brazolot Millan et al. 1999; Puri, Weyand et al. 2000). However, in human studies, blood samples are usually taken via a vein puncture in the forearm (Liao, Li et al. 1999; Baldy, de Lima et al. 2004). Another method to collect human blood samples is by collecting drops of capillary blood, obtained via a finger prick. This method was used in some studies to obtain blood samples of infants and children under the age of 5 years (Whittle, Jaffar et al. 2002). Fortunately, there is a high correlation between the

two methods of blood sampling and therefore using different methods does not pose a problem in human trials (Novello, Ridolfi et al. 1996).

Another way to measure efficacy of vaccines is by measuring CTL activity. However, this is not a common phenomenon in human epidemic studies. More often CTL activity is measured in a small set of subjects, probably due to the high amount of work which is accompanied with it. Nevertheless, several attempts have been made to specify the role of CTLs in human immunisation. This is important, because it seems that vaccines are less effective in elderly due to a decline in CTL activity. As shown in figure 11 above, comparing CTLs of healthy adults (mean age 32 years) and elderly (mean age 77 years) revealed that elderly have less activated CTLs upon immunisation against influenza, resulting in less protection (Deng, Jing et al. 2004). When trying to increase this by using another vaccine, no additional protection was offered compared to the traditional vaccination despite the increased CTL activity after immunisation (Powers 1997). The lack of additional protection was thought to be caused by the low titer of antibodies, which did not increase with the different vaccine.

The opposite was seen in a study on HIV vaccine. Despite increased antibodies after immunisation compared to a placebo, no significant increase in CTL activity was found in adults of 18 to 50 years (Cleghorn, Pape et al. 2007). Unfortunately, protection was not high, as 4 out of 160 participant contracted HIV. This illustrates that it is not predictable which arm of the immune response can offer protection and what mechanism is behind this. Therefore, it is hard to determine which parameter should be used.

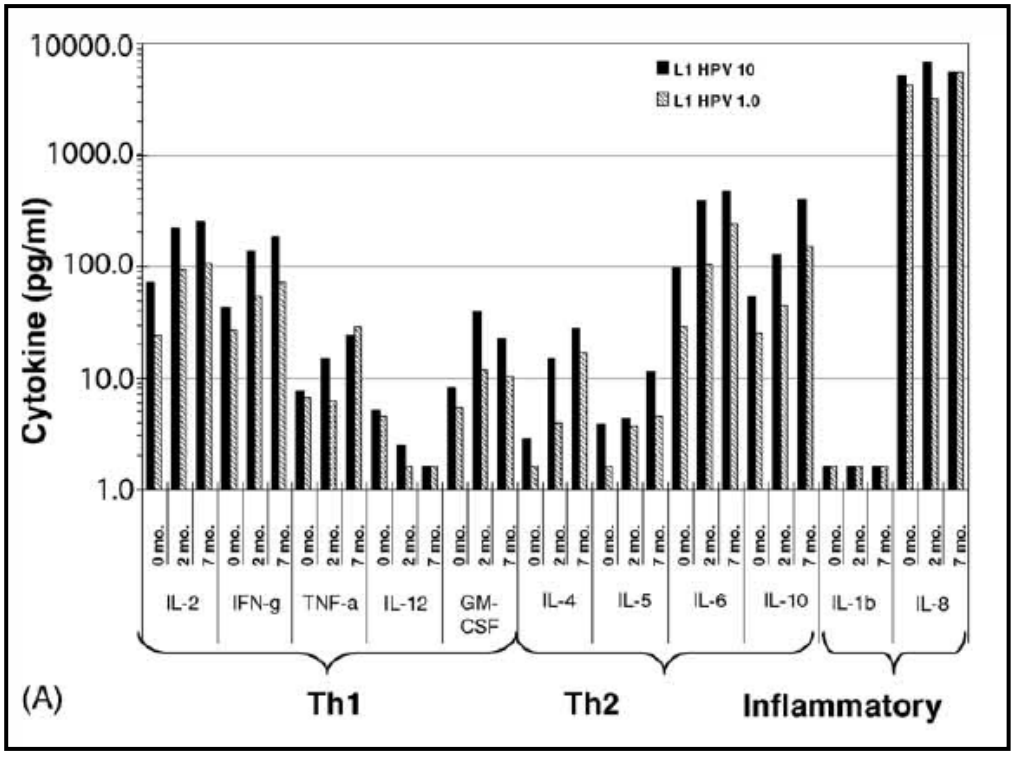

Figure 12. Cytokine production upon immunisation against HPV in women. Both T_h1 and T_h2 cytokines increase after immunisation. This indicates that an immune response has occurred, but does not specify this, making it a very limited parameter. Adapted from Pinto, Castle et al. 2005.

A way to clear up the picture can be by measuring the cytokines. However, choosing the right cytokines from a wide subset of cytokines can be difficult. A subset of 5 cytokines can already identify 32 cytokine profiles in $CD4^+$ or $CD8^+$ cells in adults (De Rosa, Lu et al. 2004). Moreover, the production of most T_h1 and T_h2 cytokines increased after vaccination for Human Papilloma Virus (HPV) in women, as shown in figure 12 (Pinto, Castle et al. 2005). This makes it a non-specific marker. Next to this, no correlation between cytokine production and specific antibodies were found, suggesting that

up-regulation of cytokines only states an increase in immune response but not specifies the mechanism behind this. Cytokine assays are therefore not an optimal device to measure efficacy of vaccines in human studies.

4.2 Factors influencing vaccine efficacy

During vaccine efficacy studies in human epidemic trials, it became apparent that factors such as genetics and age seem to have an effect on immunisation. Also lifestyle factors such as diet and smoking affect vaccine efficacy. As these factors do not occur among animals, it was noticed for the first time in human studies. Because the protection differed between populations, more research was conducted to seek the cause of these differences.

When the same lot of vaccine was used in different populations, the amount of antibodies induced differed between the populations. More detailed research showed that certain HLA class II genes were correlated to the height of response that was elicited (Poland, Ovsyannikova et al. 2001). Next to this, it was found that siblings are more likely to induce the same amount of antibodies than non-siblings (Klein, Fireman et al. 2007). Furthermore, 45% of the variability in varicella zoster immunisation came from genetic origin. A study on twins revealed that 88,5% of the variability in measles IgG is due to genetic effects (Tan, Jacobson et al. 2001). Also Single nucleotide polymorphisms (SNPs) vary within human population, which can affect the efficacy of vaccines. A common SNP in the IL-1β gene increased the variability of efficacy among HBV vaccinated humans (Yucesoy, Sleijffers et al. 2002). This shows that genetic influences can affect protection offered by vaccines, a factor that should be accounted for when applying vaccines worldwide.

Age also seems to influence the induction of protection upon immunisation. Elderly seem to have a less strong immune response after immunisation, as was mentioned before. A decreased in CTLs was found after immunising elderly compared to adults (Deng, Jing et al. 2004). This is also reflected in the prevalence of pneumococcal infection among elderly, which did not decrease after immunisation (Koivula, Sten et al. 1997). This shows that the immune system changes with age. Next to this, it was found that young children and infants show little differences in eliciting antibodies or protection. When infants were immunised at birth or at the age of 2 months, no difference in the amount of antibodies was found after 7 months (Lieberman, Greenberg et al. 1995). Also, the amount of doses does not influence the amount of antibodies elicited in infants and children, but it does induces differences in adults, as mentioned before (Naess, Aarvak et al. 1999; Whittle, Jaffar et al. 2002; Goldblatt, Southern et al. 2006). This implies that the immune system is insensitive at young age, which could be due to immaturity. It seems that the immune system is more sensitive for changes as age rises, but insensitive when it is still immature at infancy and diminishing again at old age. However, a meta-analysis showed that influenza vaccination does decrease the prevalence in infants from 6 to 71 months, children from 6 to 17 years and adults up to 60 years, but failed to do so in elderly people from 64 years and older (Nichol 2008). This implies that although the immune system is insensitive at young age, protection is induced. This however, does not imply for elderly. Therefore, the role of age and the effect on immunisation is not clear yet and more research is necessary to elucidate this.

Another factor that can play a role, especially in children, is interference between vaccines. Children receive many vaccines during infancy, some even at the same time to decrease the amount of administered doses. A consequence of this method is the possibility of interference. This can occur when several antigens are given and not against all antigens an immune response is induced. However, little interference was found when infants were immunised with 4 different antigens in one vaccine (Shinefield, Black et al. 2005). The specific antibody titers reached the criterion level of all 4 antigens, implying that protection is

offered against all pathogens. However, interference was found in a study combining 2 different antigens. Even when the antigens were given in separate injections and in separate time, interference occurred and decreased the amount of antibodies elicited (Daum, Zenko et al. 2001). Therefore, it is not certain whether proper immune memory is induced when different antigens are used at the same time, leaving people unprotected against certain pathogens.

Next to these factors, environmental factors such as stress, nutrition and smoking can also influence the efficacy of vaccines (Van Loveren, Van Amsterdam et al. 2001). Some of these factors will have a greater effect than others. Because the lifestyle of people changes worldwide time after time, it is important to be aware of these changes and taken them into account during the development of vaccines.

One such lifestyle that may affect immunity is smoking. Although there is little recent research for it, it appears that smoking does have an impact on the efficacy of vaccines, albeit it is minor. One study found that smoking even increased the antibody titers against influenza after immunisation in elderly people, although there was no difference in prevalence of the disease between smokers and non-smokers (Cruijff, Thijs et al. 1999). On the contrary, smoking was reported to be a negative predictor for immunisation against influenza in elderly (Nicholson, Kent et al. 1999). Smoking was also reported to have a negative influence on the immunisation against influenza and HBV in children from 2 months to 5 years, when they live with members in the household who smoked (Vadheim, Greenberg et al. 1992; Jafari, Adams et al. 1999). All together, there are several articles which find that smoking has a small influence, either negative or positive, but there are little recent studies done on this subject making it difficult to state the exact role.

	Seroprotected group	Nonseroprotected group	*P*
Maternal age (y)	22.1 ± 5.5	24.9 ± 5.3	.04
Smoking history	5/29 (17%)	19/35 (54%)	.005
Maternal weight (kg)	64 ± 11	75.5 ± 17.7	.003
BMI	24.9 ± 4.6	30.3 ± 8	.002
≥30	5/29 (17%)	14/35 (40%)	.04
≥34	1/29 (3%)	10/35 (29%)	.008
GA at 1st vaccination (wk)	18.6 ± 5.7	18.9 ± 4.8	NS
GA at 2nd vaccination (wk)	25.1 ± 5.8	24.6 ± 5.7	NS
Vaccination-to-rescreening interval (wk)	11.1 ± 5.1	11.5 ± 5.5	NS

BMI = body mass index; GA = gestational age; NS = not significant. Data are presented as mean ± standard deviation or proportion (%).

Tabel 2. Univariate analysis of seroprotected and nonseroprotected groups of pregnant women immused against HBV. Maternal age, smoking and BMI are factors which significantly influenced seroprotection. Adapted from Ingardia, Kelley et al. 1999 .

Another upcoming life style problem is obesity, which affects populations worldwide. There are, however, little studies done on this subject, making it difficult to predict the effect on immunisation. Some articles date more than 20 years back, but already indicating that a higher BMI has a negative influence on efficacy of vaccines (Weber, Rutala et al. 1985). Pregnant women who were obese or smoked also show declined antibody titers after vaccination for HBV, as shown in table 2, although this was tested on a small group of women (Ingardia, Kelley et al. 1999). Another study indicated that a high BMI affects mostly the antibody response (Van der Wielen, Van Damme et al. 2006). Unfortunately, it is hard to find a large scale epidemic study which investigates lifestyle factors such as obesity or smoking, therefore making it hard to prove their effect on immunisation.

It is clear that there are many unresolved issues involved in immunisation in human populations which need to be addressed and resolved before highly protective vaccines can be produced. Moreover, the human population does not remain constant when looking at life style and age, making it even more difficult to predict the outcome of a human trial.

5. Discussion

5.1 Comparing animal models and human trials

When comparing human trials and animals models, differences appear, even though many factors are taken into account during studies on efficacy of vaccines. It is clear that humans are not the same as animals, so differences are destined to come up. More important, as it is not possible to use the same methods in human trials as in animal models, it sometimes is not possible to find an explanation for these differences. Moreover, some results from animal studies cannot be verified in humans for the reason that the work can be too abundant to do large scale, or would be unethical to perform in humans. This will leave gaps in the puzzle of immunisation.

Antibody titers can give an indication of the efficacy of vaccines and is a widely used method in human trials and animal models. Unfortunately, it is not possible to challenge the participants of human trials with the pathogen, thereby making it difficult to asses the effect on the antibody titer. Even though some investigations measure antibodies after infection, usually more time has elapsed between immunisation and infection than in animal models. Because it also occurs sporadically, it is difficult to draw conclusions from it (Amanna, Carlson et al. 2007).

Another difference between animal and human research is the dosage of antigen which is used. The dosage used in animal models is relatively higher than the dosage used in human trials when compared in grams per kilogram of body weight (McCluskie, Brazolot Millan et al. 1999). This can be an important factor, because the amount of antigen can influence the type of immune response in animals, as was shown before when determining the IgG2a/IgG1 ratio of mice after immunisation (Lieberman, Clements et al. 2007). However, such

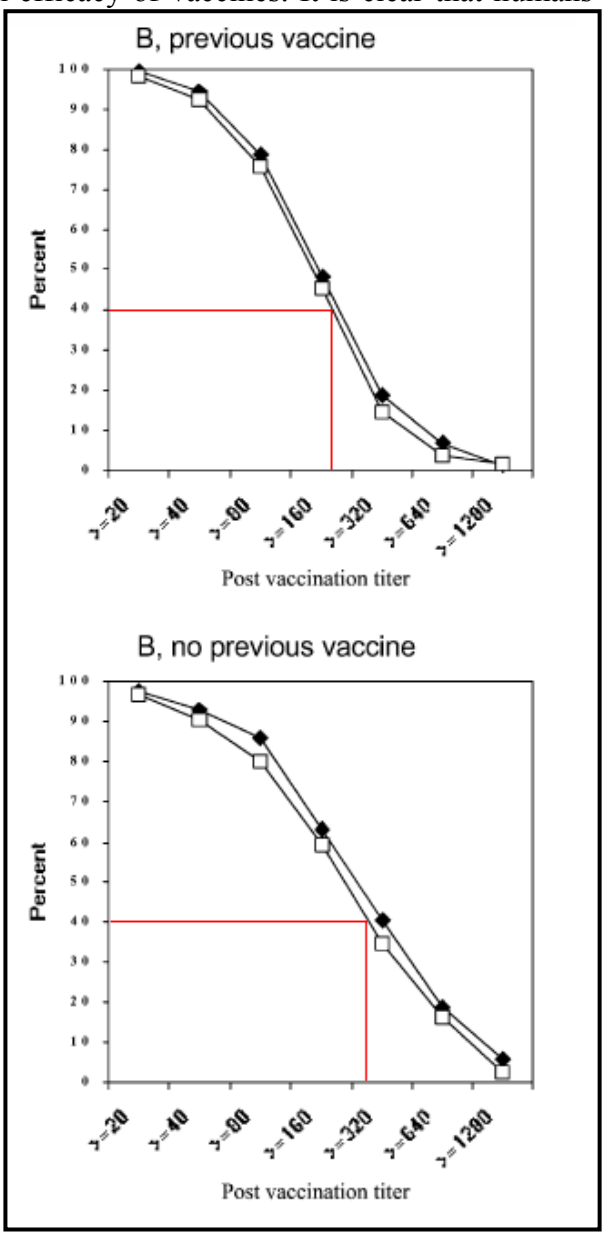

Figure 13. Percentage of subjects achieving indicated post vaccination titers in full (closed diamond) or half dose (open squares) groups. Subject having received a previous vaccine up to 3 years earlier, show lower antibody titers, as indicated as example for 40%. Modified from Treanor, Keitel et al. 2002.

differences were not seen when different amounts of antigen were used in a trial with infants. The IgG subtype did not differ between the different dosages, but the amount of cytokines did. It increased and decreased again however, with different dosages. Therefore, no correlation could be found between dosage and amount of cytokines (Vernacchio, Bernstein et al. 2002). This could be due to the lower dosage used in humans compared to animals. Nevertheless, it seems that dosage does influence the response elicited, albeit less in humans than in animals, but it remains a factor that needs to be considered when determining the dosage.

Next to these differences, contradicting results have also been found using different amount of doses to elicit antibodies in human trials. As mentioned before, an extra boost dose of antigen can increase the amount of antibodies in adults, when this is given several months after the last immunisation (Naess, Aarvak et al. 1999). This implies a sort of memory mechanism that can produce a high amount of antibodies in a short time. However, it was noticed by Treanor et al. that subjects who received a pre-vaccination up to 3 years earlier in life yielded lower titers than those who did not received pre-vaccinations, as shown in figure 13 above (Treanor, Keitel et al. 2002). This difference however, was not significant but there seemed to be a trend. Moreover, this trend was present in both groups receiving full or half dose of antigen, suggesting that pre-elicited memory has more effect on immunisation than the dose of antigen used for immunisation. Such contradicting results reveal that the precise mechanism behind eliciting antibodies and its effect on efficacy are not clear yet; therefore, more research would be necessary before it can be assessed as a defining parameter.

CTL activity is not commonly measured in human trials, but it is used more often in animal models to asses the cellular response and mechanisms behind this. It appears to be an indicating parameter in animal models when correlating CTL activity to the protection of animals after a challenge. However, in human trials, it seems that CTL activity is not always correlated to protection after immunisation, as CTL activity can be increased without having increased protection (Powers 1997). Therefore, it would be advisable to do more research on CTL activity in human trials before using it as a correlate of protection.

This also accounts for assessing efficacy via cytokine measurement. In both animal and human models, it seems that the role of cytokines is not clear yet. Contradicting results found in animal models also complicate this. As was mentioned before, $IL4^{-/-}$ and $INF^{-/-}$ mice seem to have a skewed preference to a T_h2 response after a bacterial challenge (Mills, Ryan et al. 1998; Spellberg, Ibrahim et al. 2008). However, another study found that $IL4^{-/-}$ mice have a preferred IgG2a (T_h1) response when they were immunised against Malaria (Hui and Hashimoto 2008). This presents an opposite scenario, making it difficult to extract a mechanism from it. Next to this, the role of cytokines in human is not clear either. After immunising women against HPV, the production of most T_h1 and T_h2 cytokines increased, as mentioned before (Pinto, Castle et al. 2005). This

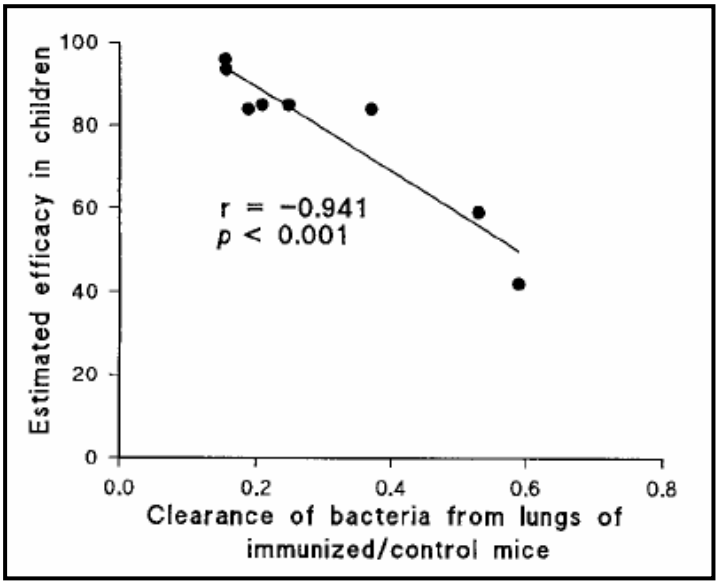

Figure 14. Correlation between bacterial clearance in mice and the vaccine efficacy in children. Lower clearance in mice is negatively correlated to efficacy in children. Adapted from Mills, Ryan et al. 1998.

shows that in human studies the role of cytokines is not clear and it seems to be a more general marker of an immune response than a specific marker. These contradicting results and the many other factors influencing the amount and type of cytokines, make it impossible to attribute a certain cytokine profile to an immune response, therefore would not be reliable to use as a parameter.

The differences between the outcomes of animal and human research could be explained by some general differences between animals and humans. Most important are the genetic differences, especially found between HLA and MHC types. This could lead to differences in response to certain diseases, giving a biased picture. Moreover, pathogens are not always as immunogenic in animals as in humans. Such differences in models are hard to subvert and can inhibit the extrapolation of data from animals to humans. Therefore, it is important to realise that animal models cannot always predict the outcome of immunisation.

To overcome such obstacles, cell models can be used. However, cell models are not without limitations either. Differences were found between in vivo and in vitro cell models. Human isolated DCs and in vitro generated DCs differ strikingly in antigen presentation skills. Also, the required stimuli to induce maturation were different (Schnurr, Chen et al. 2005). This shows that it is important to use a representative model for investigating the role of vaccines, because these differences can bias the results.

On the other hand, matching results between animal and human models can be found as well. Figure 14 above shows the result of an interesting study, which revealed that the clearance of *B. pertussis* measured in murine models correlates to the efficacy of the vaccine in the human trials when the same vaccines were used (Mills, Ryan et al. 1998). However, the immunogenicity of the vaccine was not predictable in the murine model, due to the differences between the models.

Taken together, this shows that there are differences in animal models compared to humans and it is not predicable in what manner. This makes it difficult to extrapolate data to human populations. Therefore it is important to realise that human trials will carry some risk with them because not all factors can be tested in animals.

5.2 Research pitfalls

Next to differences between animal models and human trials, there are also some general pitfalls which should be taken into account when evaluating the meaning of those studies.

A factor that can limit the reliability of research is the differences between the groups compared in human trials. Differences can occur when it is difficult to find participants or participants are lost during the follow up of a study. This can reduce the power of a study,

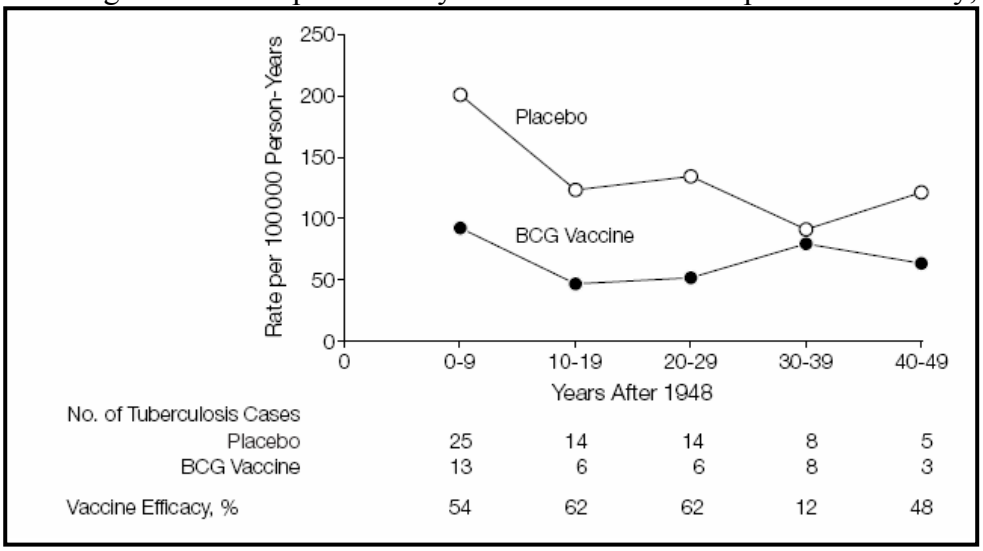

Figure 15. The efficacy of BCG vaccine and prevalence of Tuberculosis since 1948. The number of reported cases of Tuberculosis has decreased over the years, from 48 cases to 8 cases. This can bias the determining of the efficacy of the vaccine. Adapted from Aronson, Santosham et al. 2004.

thereby making it harder to find significant differences. More important, it can also bias the results, as these results could be due to differences in the studied groups. This loss of participants can occur more easily in studies were participants need to appear several times in a long period of time. One study had to exclude 639 (66.95%) participants from their study due to loss of follow up, which resulted in too small power of groups and therefore had to exclude whole groups (Baldy, de Lima et al. 2004).

Another factor that can bias the results is a decline in prevalence of a disease. Testing the efficacy of vaccines during a long follow up study can become false positive if the overall prevalence of the disease has declined. This has been reported in a 60 year follow up study on tuberculosis protection after a BCG vaccination, as shown in figure 15 above (Aronson, Santosham et al. 2004).

It can also be the case that a disease is not malignant enough, which makes the onset and progress of the disease hardly noticeable. This could lead to an overestimation of the efficacy of a vaccine, especially when measuring large scale in human populations. Also, it would be difficult to link humerol or cellular responses to the clinical phenotype of a disease if it is too benign. The clinical effect of the vaccine would also be difficult to determine. One study on swine showed that there were no differences in the welfare of the animals between control and infected swine, although high differences between CTL activity and antibodies were found (Zuckermann, Garcia et al. 2007). Because there are no clinical signs, it is not possible to determine the mechanism behind the efficacy of the vaccine, if it is effective in the first place. This can influence the interpretation of results, which may lead to false conclusions.

5.3 Factors influencing studies

Next to these specific pitfalls, there are also general factors which influence the results of studies, both in animal models as in human trials. One of those factors is age, which plays a pivotal role in immunisation, as differences are found between immunisation of infants, adults and elderly. Next to this, route of delivery also plays an important role, as different cell types are targeted with different methods. Such factors cannot be overcome when studying vaccines and therefore it is important to determine their role in immunisation.

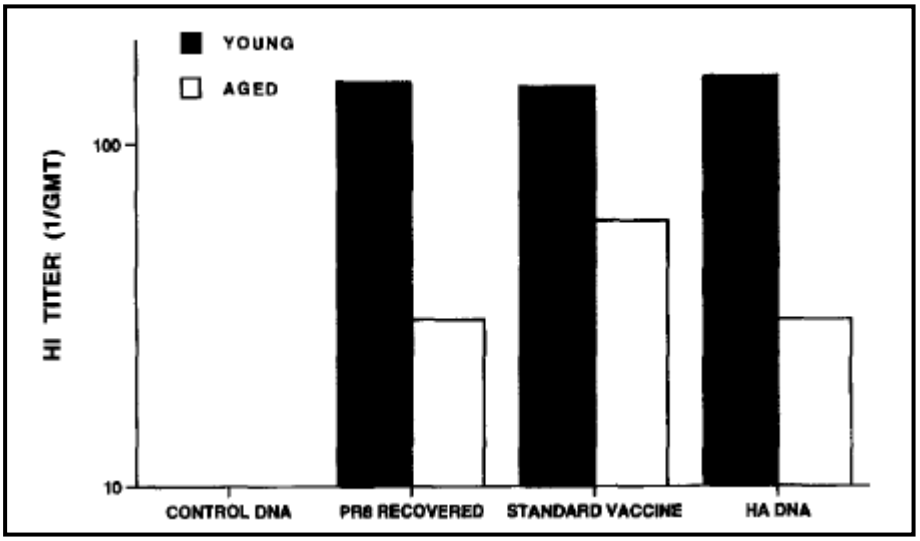

Figure 16. Antibody titers after immunisation against influenza of young and aged mice. Aged mice show diminished antibody titers after influenza immunisation. Adapted from Bender, Ulmer et al. 1998.

The effect of age on the effectivity of immunisation became apparent in both animal models as in human trial investigations. It was first seen that younger mice of 4 to 6 weeks of age produced higher titers of antibodies than older mice of 8 to 10 weeks after immunisation (Barry and Johnston 1997). This became more apparent when mice of 18 to 28 months of age

were immunised against influenza (Bender, Ulmer et al. 1998). Less antibody titers were seen after immunisation, as shown in figure 16 above, and also diminished CTL activity was found. However, after challenge, the survival of aged mice was comparable to that of the young mice. This showed that even though aged mice developed a lower immune response, the baseline for protection was still reached as the survival was as high as for young mice. Unfortunately, this is not seen in human population studies. A review by Jefferson et al. (2005) revealed that the protection for influenza was only modest in elderly people. Next to this, elderly were found to have less CTL activity (Deng, Jing et al. 2004). It was suggested that aging induces a shift in the balance of T_h1/T_h2 cytokines, which could influence the immune response (Han and Meydani 2000). Either way, it appears that older mammals somehow have a weakened immune system, which could lead to diminished protection, but this does not have to occur in the same manner for every mammal. This is important for the protection of elderly people, which are at higher risk for several pathogens.

Such differences were also found in very young mammals, albeit with diminished impact. Even though it was found that antibodies can already be elicited in 24 hour old mice and a CTL response 2 weeks after birth of mice, this does not seem to be the case for human infants (Siegrist, Saddallah et al. 1998). It takes 3 immunisations in 5 to 7 months time to elicit an antibody response which is considered to be protective in infants immunised at 0 to 2 months of age (Lieberman, Greenberg et al. 1995). Apparently, it is more difficult to elicit antibodies in humans than in mice, although this difference could be influenced by the dose of immunisation, which is relatively higher in mice than in humans. What is striking is the effect the number of doses used in immunisation has on antibody titers in young adults, which did not influence the amount of antibodies in infants, as mentioned before (Naess, Aarvak et al. 1999; Whittle, Jaffar et al. 2002). This could mean there is a more insensitive immune response elicited in infants than in adults, although this could be due to the limited amount of antibodies elicited in infants. Overall, is seems that an immature immune system at birth could play a role in immunisation, but this effect seems to be less persistent than in elderly.

The effect of route of delivery and the devices used to administer are also an important aspects in vaccination. It seems that the method of administering plays a bigger role during immunisation then was estimated on forehand. Because results seem to differ on this subject, it is difficult to asses their role in immunisation.

This became apparent during investigations on different nonhuman primates. As was mentioned before, differences were found using a needle, syringe or Biojector on *Aotus 1. lemurinus* monkeys, the latter inducing higher humoral responses (Gramzinski, Millan et al. 1998). On the contrary, no differences in CTL and antibody responses were found when *cynomologus macaques* were immunised against HIV-1 using Biojecor, mini-ject, syringe or needle (Rao, Gomez et al. 2006). Although only 6 animals per group were used, big differences would have been detected. It is possible that these differences were due to the different animal types used. If this would be the case, it would be more difficult to predict the effect it would have on humans. Therefore, it is complicated to determine the impact of this factor due to the contradicting results.

More important, the place of delivery can also influence the type of immune response, as different cell types of the body can be addressed. One type of immune cells which are the first cells involved in inducing a specific immune response are DCs. Therefore, many candidate vaccines are aimed at introducing the antigen to DCs. This can easily be done via an ID injection, which directly reaches the place where most DCs reside. This also mimics the natural way of infection and thereby seems to be an efficient route to present antigen. However, the consequence of a particular route of delivery is the induction of a certain arm of the immune system, which might not be appropriate for that particular antigen. It has been reported that DCs seem to have a preferred T_h1 response, thus diminished induction of

antibodies (Plotkin, Orenstein, Vaccines). Therefore, this should be considered when high amounts of antibodies against a certain pathogen need to be induced. On the other hand, the efficacy of live vaccines, bacterial and viral, is less influenced by the site of injection. This is though to be caused by the activation of DCs at different site due to the dissemination of viral and bacterial particles through the vascular system. Therefore, route and method of delivery are a factor playing a role in immunisation, although the extent of it needs to be investigated further.

Both age and route of delivery have an impact on immunisation, in human and in animal studies. As the effects of these factors are not clear yet, it is not possible to determine their role in immunisation. Therefore, it is important that more research is done on these topics.

5.4 Negative side of immunisation

Next to the factors influencing the efficacy of vaccines, there are also other issues which influence on the use of vaccine. Two of those factors are adverse effects and economic effects. Even though they have nothing to do with the efficacy of vaccine, they are important when applying vaccine large scale to humans. Therefore, I will mention them briefly.

Every vaccine has adverse effects, however, most are mild complaints such as fever or local soreness. Some vaccines however, can have some severe side effects such as inducing autoimmune diseases or associated viscerotropic disease, the latter being associated with vaccines against yellow fever (Khromava, Eidex et al. 2005). These effects are rare but fatal when developing. Another disease which can be caused by immunisation is the development of chronic arthritis, which was 6.2 time greater in HBV vaccinated children up to 5 years of age than in unvaccinated children (Fisher, Eklund et al. 2001). Pharyngitis and nasopharyngitis were also more common among immunised children than among unimmunised ones. It is important to weigh off the benefit versus the adversity, as a vaccine is made to improve quality of live and not to diminish it.

Next to this, economic factors also play a role when deciding to apply vaccines on large scale. Costs made by treating the disease need to outweigh the cost made by administering vaccines, otherwise no government will supply it. Also, if an epidemic outbreak would occur, most costs would be made by disease control expenditures such as health care personnel, provision and administration of immune globulin, which are higher than vaccination costs (Rosenthal 2003). Summing up these costs can make it economic beneficial to prevent an epidemic than to treat one.

6. Conclusion

When considering all these different studies, it seems that there are discrepancies in all the methods which are used. All of them show a particular side of the immune response, some better than others, but all of them have their significant pitfalls. It is surprising that many contradicting results are found among the studies and that all studies are done in such different manners. This makes it impossible to draw a general picture of the immune system and to find general immune parameters that always give a reliable correlation of protection. However, there are also similarities found among studies, which reveal more about the mechanism behind immunisation.

Measuring the specific antibody titer gives an indication of the strength of the humoral response. However, it does not disclose the cellular response induced by the vaccine. Therefore, it does not directly reveal to the efficacy of the vaccine, as protection can also be offered via the cellular arm of the response. Moreover, the amount of antibodies does not

necessarily reveal whether memory has been built up. Furthermore, the amount of antibodies elicited does not always correlate to the efficacy of vaccines. Therefore, the antibody titer indicates that a humoral immune response has occurred, whether this is effective to protect against the pathogen and if it is long lasting still needs to be determined.

These questions also remain unanswered when measuring the CTL activity. The strength of the lysis activity of CTLs can be determined, indicating the effectiveness of the cellular response. Although this seems to correlate to the protection found in animal studies, the role of CTLs upon immunisation is not clear in human trials. This makes is an unreliable parameter to assess protection after immunisation. Also, determining the CTL activity is more work than measuring the antibody titer. Therefore, the complete protection of a vaccine cannot be determined using only this technique.

To asses more about the state of immunisation, it is also possible to measure the cytokine production. However, it seems that the role of cytokines during the immune response is still elusive, in both animal and human studies. Therefore, it would be wise to first investigate more on the role of cytokines before correlating it to the protection of vaccines.

Other manners to determine the effect of vaccines, such as comparing the incidence or progress of the disease between an immunised and a control group, can indicate more about the clinical side of immunisation. However, this would reveal nothing about the mechanism of immunisation and therefore should be used concomitant. Moreover, it is not possible to use some of these methods in human trials. Therefore, they are not indicative for the efficacy in human populations.

The most revealing method to provide an accurate picture of the mechanism and efficacy of vaccines would be to combine several techniques. As mentioned above, most methods reveal only the humoral or cellular response. Therefore, the mechanism behind immunisation would become clearer if these methods were used concomitant. Furthermore, the techniques differ among the studies. It would be easier to compare the results of the studies, if the methods are more standardised.

Next to this, it differs per vaccine what parameter is useful to measure. Protection against intracellular pathogens are offered via CTLs, thus it would be necessary to measure these cells when determining the efficacy of a vaccine against such pathogens. On the other hand, some pathogens are best warded off with antibodies, thus determining antibody titers would be a representative method. However, for some vaccines it is not known what mechanism lies behind the protection. Therefore, it would be useful to combine methods to gain a complete picture of the immune response.

Testing vaccines on animals and using different methods will reveal much about the mechanisms behind immunisation. However, due to differences between animals and humans, there are still risks when extrapolating animal studies to human trials. Moreover, practical and ethical limitations for human studies will make it impossible to explain the differences between animals and humans.

General recommendations would be to optimise and standardise the methods to determine efficacy and use this globally. Correlates of protection should be used concomitant to be more specific of efficacy. Also, more research should be done to state the role of influencing factors such as age, route of delivery and lifestyle.

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